(60)

GENE REGULATION BY TARGETING PUTATIVE INTRAMOLECULAR TRIPLE HELIX

Patent Number: WO9417086

Publication date: 1994-08-04

Inventor(s): YOON KYONGGEUN (US); LU MEIQING (US)

Applicant(s):: APOLLON INC (US); YOON KYONGGEUN (US); LU MEIQING (US)

Requested Patent: ☐ WO9417086

Application Number: WO1994US00348 19940110

Priority Number (s): US19930008897 19930125

IPC Classification: C07H17/00 ; A01N43/02

EC Classification: C07H21/00C2, C12N15/11B, C12N15/11B7, C12N15/11B1, C12N15/11B1A, C12N15/11B1H, C12N15/11B2, C12N15/11B3, C12Q1/68B12

Equivalents:

Abstract

The present invention is related to oligonucleotides or derivatives thereof for decreasing transcription of a target gene having a purine-rich region of DNA that is significantly involved in transcription and has substantial mirror symmetry. The oligonucleotides of the present invention are substantially complementary to the purine stand. Certain preferred embodiments do not form a substantially stable intermolecular triple helix with the target gene in vitro at physiological pH. Other preferred embodiments are oligonucleotides having a circular or stemloop functioning structure that may form both Watson-Crick and Hoogsteen bonds with the target DNA. The invention also provides methods for preparing a composition for decreasing transcription of a target gene, methods for decreasing transcription of a target gene and methods for treating a vertebrate suspecting of having a disease involving the expression of a target gene.

What is claimed is:

1. A composition for decreasing transcription of a target gene wherein said composition is substantially complementary to a region of said gene which is significantly involved in transcription, said region comprising at least two arms further comprising inverted repeats of at least about 5 nucleotides each on each DNA strand, wherein at least about 65% of said nucleotides comprising said arms are purines on one strand, said strand constituting a purine strand, said composition comprising an oligonucleotide, or derivative for improving stability thereof, substantially complementary to at least a portion of one arm of said purine strand.

2. The composition of claim 1, wherein said region of the target gene further comprises from about 1 to about 50 nucleotides between said arms, said oligonucleotide being substantially complementary to at least a portion of one arm of said purine strand and at least a portion of the nucleotides between said arms of said purine strand.

3. The composition of claim 1 or 2 wherein the oligonucleotide comprises a stem-loop.

4. The composition of claim 1 or 2 wherein the oligonucleotide is circular.

5. The composition of claim 1 or 2 comprising at least about 8 nucleotides.

6. The composition of claim 1 or 2 wherein said oligonucleotide comprises from about 8 to about 30 nucleotides.

7. The composition of claim 1 or 2 wherein said arm of said strand is from about 8 to about 30 nucleotides long.

8. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 3.

9. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 4.

10. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 5.

11. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 6.

12. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 13.

13. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 14.

14. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 15.

15. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 16.

16. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 17.

17. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 19.

18. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 21.

19. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 23.

20. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 25.

21. The composition of claim 2 comprising the oligonucleotide of SEQ ID NO: 26.

22. The composition of claim 2 comprising the oligbnucleotide of SEQ ID NO: 28.

23. An oligonucleotide complementary to a region of a target gene wherein said region is substantially involved in transcription and which region forms an intramolecular triplex

in vitro at pH less than 7 thereby rendering a part of said region single-stranded, said oligonucleotide being substantially complementary to at least a portion of said single-stranded region.

24. A method for decreasing transcription of a target gene having a region which is significantly involved in transcription, said region comprising at least two arms further comprising inverted repeats of at least about 5 nucleotides each on each DNA strand, wherein at least about 65% of said nucleotides comprising said arms are purines on one strand, said strand constituting a purine strand, said method comprising the administration of an oligonucleotide, or derivative for increasing stability thereof, substantially complementary to at least a portion of one arm of said purine strand.

25. The method of claim 24 wherein said region of the target gene further comprises from about 1 to about 50 nucleotides between said arms, said oligonucleotide being substantially complementary to at least a portion of one arm of said purine strand and at least a portion of the nucleotides between said arms of said purine strand.

26. The method of claim 24 or 25 wherein the oligonucleotide comprises a stem-loop.

27. The method of claim 24 or 25 wherein the oligonucleotide is circular.

28. The method of claim 25 wherein said oligonucleotide is selected from the group consisting of SEQ
ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ
ID NO: 28.

29. A method of preparing a composition for decreasing transcription of a target gene comprising:
(a) selecting as a target a gene having a region which is significantly involved in transcription, wherein said region comprises at least two arms further comprising inverted repeats of at least about 5 nucleotides each on each
DNA strand, wherein at least about 65% of said nucleotides comprising said arms are purines on one strand, said strand constituting a purine strand; and
(b) synthesizing an oligonucleotide, or derivatives for increasing stability thereof, substantially complementary to at least a portion of one arm of said purine strand.

30. The method of claim 27 wherein said region of the target gene further comprises from about 1 to about 50 nucleotides between said arms, said oligonucleotide being substantially complementary to at least a portion of one arm of said purine strand and at least a portion of the nucleotides between said arms of said purine strand.

31. The method of claim 29 or 30 wherein the oligonucleotide synthesized comprises a stem-loop.

32. The method of claim 29 or 30 wherein the oligonucleotide synthesized is circular.

33. A method of treating a vertebrate suspected of having a disease involving the expression of a target gene having a region which is significantly involved in transcription, said region comprising at least two arms further comprising inverted repeats of at least about 5 nucleotides each on each DNA strand, wherein at least about 65% of said nucleotides comprising said arms are purines on one strand, said strand constituting a purine strand, comprising administering to the vertebrate an oligonucleotide, or derivatives for increasing stability thereof, substantially complementary to at least a portion of one arm of said purine strand.

34. The method of claim 31 wherein said region of said target gene further comprises from about 1 to about 50 nucleotides between said arms, said oligonucleotide being substantially complementary to at least a portion of one arm of said purine strand and at least a portion of the nucleotides between said arms of said purine strand.

35. The method of claim 33 or 34 wherein said oligonucleotide comprises a stem-loop.

36. The method of claim 33 or 34 wherein said oligonucleotide is circular.

37. The method of claim 33 or 34 wherein said oligonucleotide is selected from the. group consisting of SEQ
ID NO:2, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID
NO:28.

38. The method as in claim 33 or 34 wherein said oligonucleotide is administered ex vivo.

39. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having cancer involving the expression of c-myc comprising administering at least one oligonucleotide selected from the group consisting of SEQ ID
NOS: 2-6, and 26 or derivatives for increasing stability and delivery thereof.

40. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having cancer involving the expression of Erb-B2 comprising administering the oligonucleotide of SEQ ID NO: 13 or derivatives for increasing stability and delivery thereof.

41. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having cancer involving the expression of N-myc comprising administering the oligonucleotide of SEQ ID NO: 15 or derivatives stability and deliverv thereof.

42. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having hepatitis, comprising administering the oligonucleotie of EQ ID NO: 14 or derivatives for increasing stability and delivery thereof.

43. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having HIV, comprising administering the oligonucleotide of SEQ ID NO: 16 or derivatives for increasing stability and delivery thereof.

44. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having cancer involving the expression of EGFR, comprising administering the oligonucleotide of SEQ ID NO: 17 or derivatives for increasing stability and delivery thereof.

45. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having cancer involving the expression of K-Ras, comprising administering the oligonucleotide of SEQ ID NO: 19 or derivatives for increasing stability and delivery thereof.

46. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having an auto immune disease involving the expression of IL-2, comprising administering the oligonucleotide of SEQ ID NO: 21 or derivatives for increasing stability and delivery thereof.

47. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having HSV I, comprising administering the oligonucleotide of SEQ ID NO: 23 or derivatives for increasing stability and delivery thereof.

48. The method of claim 33 or 34 for the treatment of a vertebrate suspected of having HIV, comprising administering the oligonucleotide of SEQ ID NO: 25 or derivatives for increasing stability and delivery thereof.

GENE REGULATION BY TARGETING PUTATIVE INTRAMOLECULAR TRIPLE HELIX

Field of the Invention

The present invention is related to oligonucleotides, or derivatives thereof, for decreasing transcription of a target gene having a purine-rich region of DNA that is significantly involved in transcription and has substantial mirror symmetry. Such regions create sequences of single-stranded DNA in vitro. The oligonucleotides of the present invention are substantially complementary to a single stranded purine strand. Certain preferred embodiments do not form a substantially stable intermolecular triple helix with the target gene at physiological pH in vitro. Other preferred embodiments are oligonucleotides having a circular or stem-loop functioning structure that may form both Watson Crick and Hoogsteen bonds with the putative single-stranded target DNA. The invention also provides methods for preparing a composition for decreasing transcription of a target gene, methods for decreasing transcription of a target gene, and methods for treating a vertebrate suspecting of having a disease involving the expression of the target gene.

Background of the Invention

Practitioners have attempted to prevent transcription by targeting DNA. To date, when targeting genomic DNA, scientists have generally concentrated on the duplex structure of DNA formed by Watson-Crick base-pairing between the bases. To prevent the transcription of DNA, many scientists have targeted the DNA duplex with triplex-forming oligonucleotides (TFOs). See, for example, Cooney et al., Science 1988, 241, 456-459.

TFOs are designed to inhibit transcription by binding to double-stranded DNA in a sequence-specific manner to compete with and/or displace transcriptional factors that interact with the targeted cis-acting enhancer sequence.

However, the base-pairing involved in triplex formation is not well-understood and the stability of these structures is likewise poorly understood.

Hogan, WO 90/06934 and Froehler and Toole, WO 91/06626 provide examples that demonstrate the basic design of triplex-forming oligonucleotides. Two recognized motifs for the formation of a triple helix are known as the "CT" motif and the "GT" motif. The "CT" motif involves the use of a polypyrimidine oligomer as the triplex-forming oligonucleotide. For every GC base pair, there is a corresponding C in the TFO, while for every AT base pair, there is a corresponding T. Using this motif, the TFO is oriented in a parallel direction to the purine-rich strand of the duplex. The "GT" motif provides for the use of a G for every GC base pair and a T for every AT base pair. In this case, the TFO is oriented in an anti-parallel direction to the purine-rich strand of the duplex.

Unlike the intermolecular triplex-forming oligonucleotides of the prior art, the present invention involves the targeting of a putative single-stranded portion of a DNA sequence that is believed to result in vivo from the formation of an intramolecular triple helix within the target DNA. Such regions can be identified by their nuclease sensitivity and involvement in transcription in vitro, and by their substantial mirror symmetry, which is purine-rich on one strand. However, this structure has never been demonstrated in vivo. Thus, the approach of the present invention has never been attempted.

summary of the Invention

In one aspect, the present invention involves oligonucleotides targeted to particular regions of DNA that are believed to have a non-B form. Specifically, the present invention provides compositions for decreasing transcription of a target gene having a putative single-stranded region of DNA in vivo that is significantly involved in transcription and which region includes at least about 10 nucleotides, at least about 65% of which are purines on one strand, having substantially mirror symmetry in at least two palindromic arms having at least about 5 nucleotides each, the arms being either contiguous, or having about 1 to about 50 nucleotides between them. Certain compositions of the present invention comprise an oligonucleotide, or derivative thereof, substantially complementary to the purine strand and which does not form

a substantially stable intermolecular triple helix with the target gene as measured in vitro under physiological conditions. Other preferred compositions comprise substantially complementary circular oligonucleotides. Additional preferred compositions comprise oligonucleotides having a stem-loop functioning structure.

In another aspect, the invention involves the preparation of the oligonucleotides.

In a further aspect, the present invention involves the use of the oligonucleotides described above for the inhibition of transcription of genes having polypurine-rich sequences as described above. Hence, the oligonucleotides may be employed in the treatment of diseases involving such genes. The present invention therefore additionally provides methods for decreasing transcription of such genes, methods for preparing compositions for decreasing transcription, and methods for treating a vertebrate suspected of having a disease involving the expression of such gene. Such diseases include, for example, cancer, viral and autoimmune diseases, and diseases requiring transplantation.

Brief Description of the Drawings
Figure 1 illustrates the putative structure of H
DNA in vivo, illustrating the conformation of the helices.

H-DNA consists of an intramolecular DNA triplex region wherein pyrimidine-rich strand donates the third strand involved in Hoogsteen hydrogen bonding with the duplex. This formation causes the complementary region of the purine-rich strand to be single stranded. The two halves of the pyrimidine-rich strand involved in the intramolecular triplex are antiparallel. The pyrimidine-rich strand is shown as a black strand.

Figure 2 shows the putative H-DNA structure of the c-myc substrate representing the upstream region of the gene.

The tandem H-DNA triplex model for the nuclease sensitive element (NSE) structure is shown. An H-DNA structure is manifested using the Hoogsteen base pairing scheme. Watson and Crick base pairs are represented by dashes, T:AT
Hoogsteen base pairs by dots, and C:GC Hoogsteen base pairs by "plus" signs.

Figure 3 demonstrates the thymidine uptake of HL60 cells incubated with various oligonucleotides as described in
Example 1.

Figure 4 displays the thymidine uptake of K562 cells incubated with various oligonucleotides as described in
Example 1.

Figure 5 represents the steady state levels of cmyc and ss-actin mRNAs measured by reverse-PCR, as detected by ethidium bromide staining. HL60 cells were incubated with oligonucleotides KY10016 (SEQ ID NO:2) and KY10027 (SEQ ID
NO: 31), as described in Example 3. Total RNA was isolated from HL60 cells, diluted in a sequential manner and subjected to reverse-PCR. Lanes 1-4 represent the reverse-PCR product of HL60 cells incubated with KY10016. The number of cells in the reaction varies from 1000, 100, 10 and 1, respectively (Lanes 1 to 4). Lanes 5-8 represent the reverse-PCR product of HL60 cells incubated with KY10027. The number of cells in the reaction varies from 1000, 100, 10, and 1, respectively (Lanes 5 to 8). Lane 9 indicates the PCR product of untreated HL60 cells. Lanes 10 and 11, respectively, indicate the PCR product of HL60 cells in the presence of cmyc primers only and ss-actin primers only. The size of the
PCR products of the c-myc and ss-actin mRNAs are 326 bp and 218 bp, respectively.

Figure 6 illustrates the band shift analysis of triplex formation. Binding of various oligonucleotides to the duplex target (Table II) was assayed as described in
Example 1. In Lanes 1-4 and 9-11, the duplex targets at a constant concentration (5x10-8 M) were mixed with various concentrations of oligonucleotides in the reaction mixture containing 0.15 M NaCl, 10 mM MgC12 and 5 mM Tris-HCl at pH 7.0. In Lanes 5-8 and 12-15, the

duplex targets at a constant concentration (5x10 8 M) were mixed with various concentrations of oligonucleotides in the reaction mixture containing 10 mM Tris-HCl, 5 mM MgCl2, 1 mM spermine and 10% sucrose. Lanes 1 to 4 and Lanes 5-8 contain oligonucleotide KY10013 at various concentrations, 2x109, 2xl0 -8, 2x10-7 and 2x10 M, respectively. Lanes 9 to 11 contain oligonucleotide KY10016 at various concentrations, 2x10 2x10 7 and 2x10 -6 M, respectively. Lanes 12 to 15 contain oligonucleotide KY10016 at various concentrations, 2x10 -9, 2x10 , 2x10 -7 and 2x10 -6 M, respectively.

Figure 7 depicts the inhibition of cell growth in
LS180 the cells by the oligonucleotides of the invention as described in Example 2.

Figure 8 depicts the inhibition of cell growth in
HuH7 cells by the oligonucleotides of the invention as described in Example 2.

Figure 9 is a schematic of the formation of H-DNA when two arms comprising inverted repeats are present, and the 3 arm is involved in the Hoogsteen base pairing.

Figure 10 is a schematic of the formation of H-DNA when two arms comprising inverted repeats are present and the 5' arm is involved in the Hoogsteen base pairing.

Detailed Description of the Invention
In the context of protein binding, some scientists have reported that repeating copolymers of (dT-dC) (dA-dC) sequences can form a DNA structure in vitro having triplestranded and single-stranded regions, known as H-DNA. Htun and Dahlberg, Science 1989, 243, 1571-1576. (TC-AG), sequences are present in the genomes of both vertebrates and invertebrates and are frequently found in the 5' regulatory sequences. It is not yet known whether such structures form in vivo. H-DNA can be characterized, for example, by nuclease hypersensitivity, sensitivity to single-strandedspecific nucleases and strong purine/pyrimidine strand asymmetry. Kolluri et al., Nucleic Acids Research 1992, 20, 111-116.

Other researchers have found that a triplex is adopted by mirror repeats of G's and A's. A substantial number of purine-pyrimidine sequences have mirror repeats, or palindromic motifs, and this type of sequence corresponds well with the triplex model. Examples of simple repeating purine-pyrimidine sequences in mirror repeat that can form an intramolecular triple helix include GGA, AGAGG, GA, GGAA,
GAA, and GAAA. Wells et al., FASEB J. 1988, 2, 2939-2949.

Homopurine-homopyrimidine sequences are frequently found in upstream regulatory regions, in which they are suspected to play a function in gene expression. Wells et al., FASEB 1988, 2, 2939-2949. These sequences have been shown to adopt a non-B form structure, specifically, H-DNA in vitro, in the presence of a high degree of supercoiling or low pH, i.e., less than 7. See, for example, Mirkin et al.,
Nature 1987, 330, 495-497, Hanvey et al., Proc. Natl. Acad.,
Sci. USA 1988 85, 6292-6296, Htun and Dahlberg, Science 1989, 243, 1571-1575,
Kinniburgh, Nucleic Acids Res. 1989, 17, 7771-7778. In the H-DNA structure, half of the Watson
Crick duplex is disrupted and a donating polypyrimidine strand is folded back down the major groove of the other half of the repeat. See, for example, Figures 1, 9 and 10. The donating strand thus creates a triple helix by forming
Hoogsteen base pairs consisting of T-A-T and C+-G-C triplets.

The structure is termed "H-DNA" due to the presence of a flexible hinge between the duplexes as well as the requirements for protons in the formation of these triplets.

Htun and Dahlberg, Science 1989, 243, 1571-1576.

In the formation of these structures, longer regions participating in the formation of the triple helix confer greater stability. In the intramolecular DNA triplex, the pyrimidine-rich strand donates the third strand, leaving the complementary region of the purine-rich strand singlestranded, as shown in Figures 9 and 10. As indicated in

Figures 9 and 10, either arm of the purine strand can be involved in the Hoogsteen base pairing in the formation of the intramolecular triplex. The arms are designated "S1" and "S2", respectively, in the Figures. The complementary arms on the second strand are designated "S1 " and "S2 ", respectively. "L" designates the loop formed by the participation of S1 and S2 in the intramolecular triplex formation. The region complementary to L which forms part of the single stranded region in both instances is designated "L1". Depending upon which arm of the purine strand is involved, the single-stranded region will also include either S2 or S1 . In the Figures, Watson Crick bonds are depicted as solid lines. Hoogsteen bonds are depicted as dash lines.

The heavy arrows indicate the arms as inverted repeats on one strand. The inventors are not aware of a preferred configuration. Since the promoter region is targeted, it is not necessary that the purine-rich strand be the strand normally transcribed in the gene of interest, as shown in the examples which follow.

It has been postulated that H-DNA may be widespread in genomic DNA since TC-AG repeats long enough to hybridize with (dT-dC)18 are frequently found in human DNA. These regions are found in areas important for transcription, replication, and recombination. The formation of H-DNA in vivo could influence DNA-protein interaction and could absorb negative supercoiling generated by transcription.

H-DNA structures may be verified, for example, using reagents that provide information about DNA conformation, such as bromoacetaldehyde, chloroacetaldehyde, diethyl pyrocarbonate, 0504, and dimethyl sulfate, as discussed in Wells et al., FASEB 1988, 2, 2939-2949.

In the present invention, oligonucleotides are designed to target the putative single-stranded region of the H-DNA in an antisense manner with high stability and specificity. Alternatively, the oligonucleotides of the invention may bind to a putative transcriptional factor that interacts with H-DNA. It is believed that a family of transcriptional factors may be implicated since the transcriptionally important H-DNA sequences of several genes are very similar. Thus, a single oligonucleotide of the present invention may be able to decrease transcription of several genes implicated in, for example, cancer.

One example of a gene element having strong purine/pyrimidine strand asymmetry is the c-myc nuclease sensitive element (NSE) which has been shown to bind transcriptional factors (Kolluri et al., Nucleic Acid Res., 1992, 20, 111-116; Postel et al, Science 1993 261, 478-480), be involved in transcriptional regulation, and form H-DNA in vitro. A model has been postulated whereby two tandem triplexes are involved in the H-DNA structure, and the pyrimidine-rich strand participates in both triple helices, as illustrated in Figure 2. Firulli et al., Biochem. and Biophys. Res. Communications 1992, 185, 264-270. This element is a cis-acting positive transcription element in the case of c-myc. Davis et al., Proc. Natl. Acad. Sci. USA 1989, 86, 9682-9686.

Others have targeted c-myc using TFOs. A TFO with high affinity for c-myc is reported in Postel et al., Proc. Nat'l. Acad. Sci. USA, 1991, 88, 8227-8231 and Cooney, etal., Science, 1988, 241, 456-459. In Postel, a "G"-rich oligonucleotide, the PU1 oligonucleotide (SEQ ID NO: 30), was shown to form a triplex with substrate DNA comprising the nucleotides -153 through -117 from the P1 promoter.

Reduction in cytoplasmic c-myc P1 mRNA levels in Hela cells using PU1 were reported. Postel reported that a control sequence, comprising a "C" -rich oligonucleotide Pyl (SEQ ID NO: 2) did not form a triplex with the substrate DNA and did not reduce myc P1 mRNA levels to a statistically significant degree at the concentrations tested. Both oligonucleotides were added at concentrations ranging from 25 MM-125 ssM in the testing for mRNA reduction. The results with Pyl in Postel suggested that this oligonucleotide did not have the capability to reduce the c-myc mRNA.

In contrast, the present invention involves targeting the putative single-stranded region of the H-DNA in an antisense manner. According to the present invention, "C" -rich oligonucleotides directed against c-myc, including the Pyl sequence disclosed as ineffective in Postel, provided a surprisingly superior result in demonstrating inhibition of c-myc transcription as compared to a conventional antiparallel G-rich TFO in several cell lines, and at a 100fold less concentration. This result was particularly surprising since the occurrence of H-DNA in vivo has not yet been verified. Oligonucleotides of the present invention can be prepared for any homopurine-homopyrimidine sequences found in regulatory regions that are potentially capable of forming
H-DNA. The present invention thus provides therapeutic oligonucleotides for transcriptional regulation of many genes. Other genes having homopurine and homopyrimidine regulatory sequences include Erb-B2, K-ras, EGF receptor, IL2 receptor alpha, collagen type I, IL2, beta-actin, glucocorticoid receptor, HIV ppt, HIV LTR, HSV I and HBV ppt.

These regulatory sequences are often present upstream of the transcription initiation site, but they may also be within an intron or in the 3' untranslated sequences, for example.

The present invention specifically provides compositions for decreasing transcription of target genes such as those listed above, which potentially have a nuclease-sensitive region of DNA significantly involved in transcription and which region includes at least about 10 nucleotides, at least about 65% of which are purines on one strand, and which region has substantial mirror symmetry in at least two palindromic arms having at least about 5 nucleotides each, the arms being either contiguous or having about 1 to about 50 nucleotides between them. Nuclease sensitivity is not required. Several different means for selecting the target gene can be employed and are described below.

Nuclease sensitivity is defined as at least about 20% greater than average sensitivity to nucleases such as
DNase I and S1, and preferably at least about 50% greater than average. Average sensitivity may be measured, for example, by subjecting isolated nuclei to DNase digestion and probing with a radiolabeled DNA fragment in an area of a gene that is not actively transcribed. See, for example, Postel et al., Proc. Natl. Acad. Sci. USA 1991, 88, 8227-8231.

Nuclease sensitivity reflects an alteration of chromatin that results in the disruption of normal nucleosome structure. See, for example, Larsen and Wientraub, Cell 1982, 29, 609-622. This may be directly related to gene transcription because nucleosomes restrict transcriptional activity. Indeed, there appears to be a correlation between the hypersensitivity and transcriptional activity. For example, the DNase hypersensitive site of the c-myc upstream region disappears in HL60 cells coincident with the cessation of c-myc transcriptional initiation. This region of the cmyc gene has been shown to be required for a high level of cmyc gene expression and to bind several nuclear factors.

See, for example, Davis et al., Proc. Natl. Acad. Sci. USA 1989, 86, 9682-9686. The present invention thus provides the advantage of binding to a region capable of forming a non-B DNA structure, which may be present only during active transcription.

An area of the gene that is significantly involved in transcription is defined as a region that has been found, using molecular genetics, to be important in the initiation and/or enhancement of transcription, and/or à region where transcriptional factors bind or interact. An example of a test that may be performed to determine whether a region is transcriptionally important is a nuclear run-on assay.

Groudine et al., Molecular Cellular Biology, 1981, 1, 281288. Additionally, conventional molecular genetics may be used to determine whether a region of DNA is involved in transcription by, for example, enhancer mapping, which involves linking the region to a reporter gene to measure its transcription with and without the presence of the DNA.

One example of an enhancer mapping assay is a CAT assay where the CAT gene is coupled with the region suspected to be involved in transcription. In preferred embodiments, upon

comparison of CAT expression with and without the DNA region potentially involved in transcription, at least about a 50% reduction in CAT expression in the absence of the DNA element denotes significant involvement in transcription.

More preferred embodiments of the invention entail DNA regions whose absence result in at least about a 75% reduction in CAT expression, and most preferred embodiments demonstrate at least about a 90% reduction in CAT expression.

Preferably, the target region is a putative singlestranded region formed due to an intramolecular triple helix created by substantial mirror symmetry within a purine-rich or a pyrimidine-rich segment. Substantial mirror symmetry is defined as a sequence having at least 2 arms constituting inverted repeats. The inverted repeats need not be a perfect match so long as there is substantial symmetry, preferably with at least about 75% of the bases within the arms exhibiting mirror symmetry. The arms may be contiguous or non-contiguous and are each preferably at least about 5 nucleotides long, and more preferably about 5 to about 30 nucleotides long. Those sequences that are non-contiguous preferably have about 1 to about 50 nucleotides between the two arms. Such intramolecular triple helices are stabilized in vitro by a pH lower than 7 and negative supercoiling. The length of the purine-pyrimidine tract involved also determines in vitro stability. In vivo, protein binding may also provide stability. See Wells et al., FASEB 1988, 2, 2939-2949. In fact, it has been suggested that certain proteins may act as positive regulators of transcription by altering DNA topology in favor of the H form. See Davis et al., Proc. Natl. Acad. Sci. USA 1989, 86, 9682-9686.

Preferably, the putative single-stranded target region is from about 8 to about 30 nucleotides long, and comprises at least about 65% guanines and adenines.

The compositions of the invention comprise an oligonucleotide, or derivative thereof, substantially complementary to at least a portion of the area representing one of the arms and, in certain embodiments, the region between the arms, if any, on the purine strand. The oligonucleotide does not form a substantially stable intermolecular triple helix with the target gene as demonstrated by in vitro testing.

Intermolecular triple helix formation with the target region is measured at conditions comprising physiological pH and can be detected by routine testing available to one skilled in the art. One example of such a test is mobility shifting upon gel electrophoresis. The triple helix species migrate more slowly than the double helix species. The equilibrium constant of the ratio of duplex to triplex formation can be measured by quantitation of each species. Upon electrophoresis, an equal intensity between the duplex species and the triplex species defines the equilibrium constant. An equilibrium constant less than 106, for example, indicates weak affinity for triplex formation, further indicating that a substantially stable intermolecular triple helix does not form.

In certain preferred embodiments, the oligonucleotides of the invention comprise at least about 8 nucleotides, preferably about 8 to about 30 nucleotides. In a further preferred embodiment, the oligonucleotides of the invention comprise at least about 65% cytosines and thymines.

In certain embodiments, the oligonucleotides of the invention are circular. Researchers have found that circular oligodeoxyribonucleotides bind to single-stranded target regions more strongly than linear DNA complementary sequences. This greater stability is apparently due to the presence of Hoogsteen and Watson-Crick binding domains in the circular oligonucleotide. Thus, a circular oligonucleotide may form an intermolecular triple helix with the putative single-stranded region of the target gene. See, for example, Prakash and Kool, J. Chem. Soc., Chem. Commun. 1991, 11611163 and Figure 3.

In other embodiments, the oligonucleotides of the invention have a stem-loop functioning structure. The stemloop functioning structure is defined as a sequence that has two arms having substantial mirror symmetry connected by a linker. The stem-loop functioning oligonucleotide thus can form an intermolecular triple helix with a complementary single-stranded DNA. Like circular oligonucleotides, oligonucleotides having a stem-loop functioning

structure may form both Watson-Crick and Hoogsteen bonds with a singlestranded target. See, for example, Giovannangeli et al., J.

Am. Chem. Soc. 1991, 113, 7775-7777. The length of the stems of such structures are preferably at least 5 nucleotides.

The loop is at least 3 nucleotides long, and preferably less than 10. In a preferred embodiment, the loop is 3-7 nucleotides. Jaeger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 7706-7710 (incorporated herein by reference). The linker may be any of a number of compounds, as would be understood by one skilled in the art, once armed with the present disclosure. Such compounds include, for example, nucleotides, polyethylene glycol, polyamides, and methylphosphonates.

Whether it is linear, circular, or a stem-loop functioning structure, the oligonucleotide need not match the target sequence exactly; it may span only a portion of it and some mismatches are contemplated. The phrase "at least a portion of" represents preferably at least about 8 nucleotides of the target DNA. The length of the oligonucleotide is preferably from about 8 to about 30 nucleotides. The oligonucleotides should be a size which is long enough to bind specifically to the target region, but not too large to prohibit entry into a cell. The number of mismatches contemplated are those which do not prevent binding of the oligonucleotide to the extent necessary to reduce transcription.

While any length oligonucleotide may be utilized, it is preferable to use a length that confers specificity.

The length of the oligonucleotide required for specificity depends upon the content of the bases, as discussed, for example, in Herschlag, Proc. Natl. Acad. Sci. USA 1991, 85, 6921-6925. Specifically, it has been demonstrated that adding more bases to an oligonucleotide may decrease specificity if binding to the target becomes too strong, thus also allowing binding to sequences other than the target desired. Alternatively, in some cases, shorter sequences may be less specific in hybridizing to the target and may be more easily destroyed by enzymatic degradation. Further, it is known that GC base pairs bind more tightly than AT base pairs. Hence, in the present invention, when the purine strand is G-rich, oligonucleotides having at least about 8 nucleotides are preferred.

In addition, the size of the oligonucleotide is limited by its ability to enter the target cell. Large oligonucleotides may be somewhat less effective in interfering with expression because of decreased uptake by the target cell. It will be understood that interference with expression means that there is a detectable decrease in transcription.

The phrase "substantially complementary" refers to an oligonucleotide designed to generally have a C for every
G, a G for every C, an A for every T and a T for every A in the purine-rich strand. It will be understood by one skilled in the art that the oligonucleotide need not be perfectly complementary to the purine strand to be capable of decreasing transcription. Some mismatches can be included, so long as there remains a detectable decrease in transcription of the target gene.

It will also be understood by one skilled in the art, once armed with the present disclosure, that the oligonucleotides of the present invention differ from traditional antisense oligonucleotides targeted against mRNA.

Rather, the oligonucleotides of the invention are "antigene." In the case of the eukaryotic gene targets, since the targeted region in the DNA is a transcriptional regulatory region, this area of the DNA is not transcribed into a corresponding mRNA. In the case of viral gene targets, however, it will be understood by one skilled in the art, once armed with the present disclosure, that the same oligonucleotide of the invention may be capable of both decreasing transcription and performing an antisense function by binding to the corresponding mRNA and thus decreasing expression also.

The oligonucleotide can be a synthetic deoxyribonucleotide or a ribonucleotide, or derivative thereof, but is preferably a deoxyribonucleotide. The use of ribonucleotide derivatives has been reported by Shibahara et al., Nucleic Acids Research 1989, 17, 239-252.

Ribonucleotides may confer more binding stability than deoxyribonucleotides, as discussed, for example, in Roberts and Crothers, Science 1992, 258, 1463-1466. However, deoxyribonucleotides are generally more stable to nuclease attack.

The oligonucleotides of the present invention can be synthesized by any of the known chemical oligonucleotide synthesis methods. See for example, Gait, M.J., Ed. (1984), Oligonucleotide Synthesis (IRL, Oxford). The oligonucleotides may also be synthesized through recombinant expression from an appropriate vector.

The use of derivatives of the oligonucleotides known in the art is also within the scope of the present invention, including derivatives such as methylphosphonates, phosphotriesters, phosphorothioates and phosphoroamidates.

Additionally, beta-anomers may be replaced with alphaanomers. Sun et al., Triple-Helix Formation by a
Oligodeoxynucleotides and a Oligodeoxynucleotide-Intercalator
Conjugates, Proc. Natl. Acad. Sci. 1991, 88, 6023-6027. See also C.A. Stein & J.S. Cohen, Oligodeoxynucleotides as
Inhibitors of Gene Expression: A Review, Cancer Research 1988, 48, 2659-2668. Further, PNA (polyamide nucleic acid) may be used. See, for example, Nielsen et al., Science 1991, 254, 1497-1500. Derivatives increasing the stability of the oligonucleotides in vivo are preferred.

Other oligonucleotide derivatives include the use of linkers attached to the 5' and/or 3' termini along with a modifying group selected for its ability to damage DNA, such as an intercalator. See, for example, Nguyen et al., U.S.

Patent No. 4,835,263, which is hereby incorporated by reference.

In addition, labeling groups such as psoralen, chemiluminescent groups, cross-linking agents, intercalating agents such as acridine, or groups capable of cleaving the targeted position of the targeted DNA such as molecular scissors like o-phenanthrolinecopper or EDTA-iron may be incorporated in the oligonucleotides. See, for example,
Ts'o, WO 90/15884, which is hereby incorporated by reference.

DNA modifying groups may also be used to derivatize the oligonucleotides of the invention. Such groups include groups that cross-link, alkylate, cleave, degrade, or otherwise inactivate or destroy the DNA target or a portion thereof, and thereby irreversibly inhibit the function and/or expression of that sequence. See, for example, Ts'o, WO 90/15884. One example of a derivative that intercalates and induces covalent modifications of the target within a triple helix structure, thus increasing triplex stability, is benzo (e) pyridoindole.

The oligonucleotides of the invention may also be modified, for example, by covalent binding on either the 3' or the 5' end to a ligand or a ligand mimic for receptormediated endocytosis. Alternatively, the oligonucleotides may be mixed with such a ligand. For example, the oligonucleotides of the invention may be substituted on the 3' end with a thiol coupled to 6-phosphomannosylated proteins via a difulside bridge according to Bonfils et al., Nucleic Acids Res. 1992, 4621-4629. Such modifications would presumably increase cellular uptake and, thus bioavailability.

The term "oligonucleotide" as used herein includes both ribonucleotides and deoxyribonucleotides, and includes molecules which may be long enough to be termed "polynucleotides." Oligodeoxyribonucleotides are preferred since oligoribonucleotides are more susceptible to enzymatic attack by ribonucleotides than deoxyribonucleotides. It will also be understood that the bases, sugars or internucleotide linkages may be substituted or chemically modified by methods known in the art. Modifications may be made, for example, to

improve stability and/or lipid solubility. For instance, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting a methyl group or sulfur atom for a phosphate oxygen in the internucleotide phosphodiester linkage. The phosphorothioates, in particular, are stable to nuclease cleavage and soluble in lipid.

It is generally preferred to administer the oligonucleotides and their derivatives to an affected individual in accordance with this invention internally, such as orally, intravenously or intramuscularly. Other forms of administration, such as transdermally, topically or intralesionally may also be useful. Inclusion in suppositories may also be useful. Use of the oligonucleotides and their derivatives in prophylaxis is also contemplated. Use of pharmaceutically acceptable carriers is preferred for some embodiments.

Pharmaceutical compositions of this invention comprise a pharmaceutically acceptable carrier or diluent and an effective quantity of one or more of the oligonucleotides or their derivatives, or an acid or base salt thereof. The carrier or diluent can take a wide variety of forms depending on the form of preparation desired for administration, e.g., sublingual, rectal, nasal, oral, or parenteral.

In preparing the compositions in oral dosage form, any of the usual pharmaceutical media can be employed, for example, waters, oils, alcohols, flavoring agents, preservatives, and coloring agents, to make an oral liquid preparation (e.g., suspension, elixir, or solution) or with carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, and disintegrating agents, to make an oral solid preparation (e.g., powder, capsule, or tablet).

Controlled release forms or enhancers to increase bioavailability can also be used. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are employed. If desired, tablets can be sugar coated or enteric coated by standard techniques.

For parenteral products, the carrier will usually be sterile water, although other ingredients to aid solubility or as preservatives can be included. Injectable suspensions can also be prepared, in which case appropriate liquid carriers and suspending agents can be employed. The oligonucleotides can be combined with a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solution of dextrose, and the like.

The oligonucleotides and their derivatives can also be administered locally at a lesion by topical application of a solution or cream.

Alternatively, the oligonucleotides or their derivatives can be administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describes methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is provided by G. Gregoriadis, Drug Carriers in Biology and Medicine, Academic Press, 1979, "Liposomes", Chap. 14, pp. 287-341. Microspheres formed of polymers are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the nucleotides or their derivatives can be incorporated therein and the microspheres, or composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patents Nos. 4,906,474, 4,925,673 and 3,625,214.

In addition to administration with conventional carriers, the oligonucleotides may be administered by a variety of specialized oligonucleotide delivery techniques.

For example, oligonucleotides have been successfully encapsulated in unilameller liposomes. Reconstituted Sendai virus envelopes have also been used successfully to deliver

RNA and DNA to cells. Arad et al., Biochem. Biophy. Acta.

1986, 859, 88-94. Additionally, oligonucleotides can be carried into the cell by exploitation of folate receptormediated endocytosis. Leamon and Low, Proc. Nat '1 . Acad.

Sci. 1991, 88, 5572-5576.

It will also be understood that the oligonucleotides can be administered by vector-mediated delivery. Such delivery systems are within the scope of one skilled in the art once armed with the present disclosure.

Preferred methods of gene therapy include, for example, the incorporation of the gene encoding the therapeutic oligonucleotide into a retroviral vector, followed by selection of cells expressing the gene. Using the retroviral vector, the gene is then transferred into the stem cells of the patient's bone marrow ex vivo. In order to effect repopulation, the patient's own bone marrow is treated, for example, with irradiation, chemotherapy or ablation. The treated bone marrow is then transplanted into the patient.

For examples of vector-mediated delivery systems, see,
Rosenberg, et al., New Eng. Jour. Med. 1990, 570-578; Roux, et al., Proc. Natl. Acad. Sci. 1989, 86, 9079-9083; DeMonte, et al., Proc. Natl. Acad. Sci. 1990, 87, 2941-2945; Hantzopoulos, et al., Proc. Natl. Acad. Sci. 1989, 86, 35193523 and Kashani-Sabet, et al., Antisense Res. and Dev. 1992, 2, 3-15.

The oligonucleotides of the invention can also be delivered locally in vivo in high concentrations, for example, in a pluronic gel which has been shown to allow for local treatment when applied during surgery. Simons et al.,
Nature 1992, 359, 67-70.

The criteria for assessing response to therapeutic modalities employing these oligonucleotides and, hence, effective dosages of the oligonucleotides of this invention for treatment, are dictated by the specific conditions and will generally follow standard medical practices.

Overall, it is preferred to administer to patients the oligonucleotides, or derivatives, to patients in either native form or suspended in a carrier medium, in amounts and upon treatment schedules which are effective to reduce the symptomology of disease. It is within the scope of a person skilled in the art to determine optimum dosages and treatment schedules for such treatment regimens. The oligonucleotides can be administered in an amount effective to reduce transcription of the target gene. The actual dosage administered may take into account the size and weight of the patient, whether the nature of the treatment is prophylactic or therapeutic in nature, the age, health and sex of the patient, the route of administration, and other factors.

It is also possible to administer the oligonucleotides ex vivo by isolating white blood cells from peripheral blood, treating them with the oligonucleotides, then returning the cells to the donor's blood. Ex vivo techniques have been used n the treatment of cancer patients with interleukin-2 activated lymphocytes. Rosenberg et al.,
New England Journal of Medicine, 1990, 323, 570-578 (incorporated herein by reference).

The oligonucleotide therapeutics can be administered in amounts effective to decrease transcription of genes that are overexpressed while maintaining the viability of normal cells. Such amounts may vary depending on the nature and extent of the disease, the particular oligonucleotide utilized, the relative sensitivity of the disease to the oligonucleotide, and other factors.

It will be understood that the treatments of the present invention can be combined with conventional therapies, which can be administered sequentially or simultaneously.

The present invention also provides methods for decreasing transcription of a target gene

having a region of
DNA that is significantly involved in transcription and which region includes at least about 10 nucleotides, at least about 65% of which are purines on one strand and which region has substantial mirror symmetry in at least two palindromic arms having at least about 5 nucleotides each, the arms being either contiguous or having about 1 to about 50 nucleotides between them. A preferred method of the invention comprises the administration of an oligonucleotide or derivative thereof substantially complementary to one of the palindromic arms of the purine strand and, in some embodiments, the sequence between the arms when they are not contiguous, and which does not form a substantially stable intermolecular triple helix with the target gene at physiological pH.

In preferred methods, a target gene is selected in which the gene has a region of DNA that is significantly involved in transcription and which comprises at least about 8 nucleotides, at least about 65% of which are purines on one strand. In other preferred embodiments, the oligonucleotide is circular. In further preferred embodiments, the oligonucleotide has a stem-loop functioning structure.

Further, the present invention provides methods of treating a vertebrate having a disease involving the expression of a target gene having a purine-rich region of
DNA that is significantly involved in transcription as described above. These methods comprise administering to the vertebrate an oligonucleotide of the invention. In certain preferred embodiments, the oligonucleotide is administered in an a blood-level concentration corresponding to an in vitro concentration of about 1 ZM.

Certain preferred embodiments of the present invention involve target genes that are oncogenic and the oligonucleotides of the invention may be used as antiproliferative therapeutics in the treatment of cancers involving the expression of the target gene(s). Other preferred embodiments entail target genes that are expressed upon virus infection and the oligonucleotides of the invention may be used to decrease the transcription of the viral genes and viral replication in the treatment of infection. Additional preferred embodiments pertain to target genes involved in auto immunity and transplant rejection.

A major advantage over the conventional triplex approach conferred by the present invention is higher affinity to the target since a putative single-stranded portion of DNA is targeted. In the intermolecular triplexforming approach, the targeted region is double-stranded.

Triplex binding is less stable than duplex binding--in contrast to stem loop oligonucleotide binding to a single stranded target, which involves Watson-Crick binding as well as Hoogsten base pairing and, thus, both specificity and stability are higher than Watson-Crick Base binding alone.

An additional advantage is the effect on only transcriptionally active regions since H-DNA appears to exist only during active transcription, according to DNase I hypersensitive site mapping. Thus, oligonucleotides of the present invention targeting regions capable of forming a putative H-DNA structure in vivo are expected to have a substantial effect only when the gene is transcriptionally active.

Another advantage conferred by the present invention is the lower concentration of oligonucleotide which can be used to inhibit transcription since, unlike the conventional antisense RNA approach, the target is a singlecopy DNA sequence. For example, tests have shown that certain oligonucleotides of the invention may be capable of decreasing transcription of a target gene in tissue culture cells in concentrations as low as 1 ZM, and even as low as .1 ssM, as seen in Figure 4a. It is believed that modifications to the oligonucleotides will decrease the required concentration even further. Contrastingly, Postel, et al., using TFOs, reported using concentrations in the 25-125 MM range to inhibit transcription in cells culture.

The following non-limiting examples are meant to illustrate several embodiments of the invention. The sequences of the genes i the examples are available, for example, from GenBank (IntelliGenetics, Mountain View, CA).

C-myc
C-myc is an example of a gene having a purine-rich region with substantial mirror symmetry. The upstream sequences of this gene have two potential sites of H-DNA formation, as illustrated in the mirror symmetry present in nucleotides 12-17 and 22-27 as well as 34-41 and 49-56 of SEQ
ID NO: 1. The expression of c-myc has been implicated in many cancers including leukemias. Examples of oligonucleotides of the invention targeted against c-myc include SEQ ID NOS: 2-6 and SEQ ID NOS: 26-27. These oligonucleotides, and their derivatives, can be used, for example, in the treatment of many cancers including carcinoma, endothelioma, myeloid leukemia and breast cancer, and more generally, any disease involving the expression of c-myc. Examples 1-4 discussed below address a c-myc target.

Materials and Methods
Cells Lines and Transfection by Lipofectin.

HL60 and K562 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. All reagents used for tissue culture were obtained from Gibco Laboratories.

Transfection was carried out using lipofectin reagent (Bethesda Research Laboratories). Various concentrations of oligonucleotides were mixed with 2 pg of lipofectin in OPTIMEM (Bethesda Research Laboratories) and added to the 96well plate containing 100,000 HL60 cells in a total volume of 100 1 per well such that the final concentration of oligonucleotide ranged from 0.1 to 5 ssm. Cells were incubated with the oligonucleotide/lipofectin mixture for 4 hours, supplemented with FBS to 10%, and harvested 24 hours post transfection.

The adherent cell lines LS180 and HuH7 were grown in alpha MEM supplemented with 10% fetal bovine serum.

Transfection was carried out with modifications from the procedure described above. Various oligonucleotides were mixed with 2 Mg of lipofectin in OPTIMEM and added to the 96well plate containing the oligonucleotide 5000 cells per well in 100 1. The concentration ranges of oligonucleotides were as above. Cells were incubated with the oligonucleotide/lipofectin mixture for 4 hours, medium was aspirated, and cells were refed with medium containing the same concentration of the oligonucleotides as initially, supplemented with FBS to 10% for 5 days, after which cells were harvested. Further additions were made to the cells during that time as required due to evaporation effects.

Thymidine Uptake and Cell Counts.

Cellular uptake by H660 and K562 cells was determined by performing an assay measuring thymidine uptake.

After the H660 and K562 cells were treated with lipofectin, 0.5 ssCi of 3Hthymidine (New England Nuclear, specific activity'= 6.7 Ci/mmol) was added to each well and incubated for 24 hours before harvesting cells. The incorporation of H thymidine into cells was measured in triplicate by an automated cell harvester and beta plate reader (Pharmacia).

Cell counts of H660 and K562 cells were performed by counting viable cells using trypan blue dye exclusion and an hemocytometer.

MTT Assay.

Cellular uptake by LS180 and HuH7 cells was determined by performing an MTT assay. Prior to harvest,
LS180 and HuH7 cells were incubated with 0.5 mg/ml MTT (3 (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl-2H-tetrazolium bromide) for 2 hours. The medium was aspirated and 100 1 dimethylsulfoxide was added to each well. The plates were shaken for 30 minutes using a plate shaker at room temperature. The absorbance was then read immediately at a wavelength 540 nm with a Vmax microtiter plate reader.

(Molecular Dynamics).

Quantitation of c-myc mRNA and ss-actin mRNA by Reverse PCR.

Messenger RNA levels of c-myc and ss-actin were analyzed as follows. Cells were incubated with various oligonucleotides under identical conditions as described above, lysed in 0.5% NP40 solution containing 140 mM NaCl, 1.5 mM MgCl2 and 10 mM Tris-HCl at pH 8.0. After lysis,
RNasin was immediately added to 1000 U/ml. RNA was then reverse-transcribed by using 400 units of Moloney murine leukemia virus reverse transcription (RT) and 0.2 pg of random primer for 1 hour at 420C. The resulting cDNA fragment was amplified with 5 units of Thermus aquaticus (Taq) polymerase in the presence of 15 mM synthetic primers specific for each of C-myc and ss-actin mRNA. Each specific primer was selected according to the published sequences and to produce amplified DNA of different sizes. Table I lists the sequence of each primer used. To quantitate the mRNA, cell lysates were diluted in a sequential manner and subjected to a quantitative PCR amplification. See, for example, Wang et al., Proc. Natl. Acad. Sci. 1989, 86, 9717 9721. A trace amount of 5'-labelled oligonucleotide was added for each reaction and the radio-labelled bands were quantitated by PhosphorolImager.

TABLE I
SEQUENCE OF PRIMERS UTILIZED IN THE REVERSE-PCR EXPERIMENT
PRIMERS SEQUENCE SEQ ID NO: c-myc 5' primer
AGCAGCGACTCTGAGGAGGAACAAGAA 32 c-myc 3' primer
AGCAGGATAGTCCTTCCGAGTGGA 33 ss-actin 5' primer
GCATGGAGTCCTGTGGCATCCACG 34 ss-actin 3' primer
CTAGAAGCATTTGCGGTGGACGAT 35
Tests of Binding to Duplex substrate.

Duplex formation was effected by combining equimolar concentrations of oligonucleotides in a buffer containing 0.15 M NaC1, 10 mM MgC12, and 5 mM Tris-HCl, pH 7.0, heating at 700C for 15 minutes, slowly cooling, and then incubating at various temperatures. Various concentrations of oligonucleotides were added to a fixed concentration of duplex target and incubated overnight. The concentration of duplex was constant at 5x10-8 M in each reaction in a total volume of 10 1. Concentrations of oligonucleotides ranged from 2x10 9 M to 2x10 -6 M. Electrophoresis was carried out on 128 polyacrylamide gels (17x18x0.8 cm) in a buffer containing 50 mM boric acid, 5 mM NgCl2 and 50 mM Tris-HCl, pH 8.3 at room temperature. In general, the electrophoresis was performed at a constant voltage of 10 V/cm with a current of 20 to 30 mA. After electrophoresis was halted, the gel was dried and the radioactivity contained in each band was quantitated using a PhosphorolImager.

EXAMPLE 1
Inhibition of Cell Growth in HL60 and K562 Cells by
Oligonucleotides.

The upstream regulatory region of the c-myc gene, beginning at -125 bp from the initiation of transcription, contains a region of high purine/pyrimidine sequence asymmetry. This region is a nuclease sensitive element (NSE) which has been shown to bind transcriptional factors, be involved in transcriptional regulation, and form H-DNA in vitro. A model has been postulated whereby two tandem triplexes are involved in the H-DNA structure, and the C-rich strand participates in both triple helices, as illustrated in
Figure 2. Firulli et al., Biochem. and Biophys. Res.

Communications 1992, 185, 264-270. This C-myc element is a cis-acting positive transcription element. Davis et al.,
Proc. Natl. Acad. Sci. USA 1989, 86, 9682-9686.

Several oligonucleotides were tested in the present assay for their ability to inhibit c-myc transcription. The oligonucleotides tested are listed in Table II and comprised the following: 1)

G-rich oligonucleotides designed to bind the duplex target in antiparallel manner (G:GC, A:AT or
T:AT); 2) G-rich oligonucleotides with a scrambled sequence; 3) C-rich oligonucleotides complementary to the target duplex
DNA in parallel or antiparallel manner (C+:GC); and 4) C-rich oligonucleotides with a scrambled sequence. An "(AP)" preceding the oligonucleotide designation indicates antiparallel orientation. As "(P)" indicates parallel orientation. The c-myc substrate comprised two inverted repeats of two arms each. The regions of symmetry comprising the arms of the inverted repeats on the pyrimidine-rich strand are underlined in the substrate sequence (SEQ ID NO: 1) and designated "S1", "S2", "T1" and "T2" respectively.

They comprise nucleotides 12-17 (S1) and 22-27 (S2); and34- 41 (T1) and 49-56 (T2). The putative in vivo purine single stranded region is highlighted and designated "Loop 2". The configuration of these regions in the triplex structure is depicted in Figure 2. The regions in the oligonucleotides complementary to Loop 2 (or its complement, as in the case of PUI) are highlighted in a similar manner. Oligonucleotides were incubated with HL60 and K562 cells at concentrations ranging from 0.5 to 5 pM, in the presence or absence of lipofectin, for 4 hours in OPTI-MEM prior to the addition of bovine serum. Thymidine uptake was measured 24 hours afterwards, as described above.

TABLE II
CIT RICH C-MYC UPSTREAM REGION
SUBSTRATE SEQ ID NO:
5'TGAGTCTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCATAAGCGCCCCTCCC3'1
S1 S2 T1 T2
3'ACTCAGAGGAGGGGTGGAAGGGGTGGGAGGGGTGGGAGGGGTATTCGCGGGGAGG
G5'
Loop 2
G-RICH OLIGONUCLEOTIDES
PU1 (POSTEL) 5' GGTTGGGGTGGGTGGGGTGGGTGGGGT 3' (AP)KY1001330
G SCRAMBLE 5' GGTGGAGGGTGGAGGGTGGAGGGTGGG 3' (AP)KY1002731
C-RICH OLIGONUCLEOTIDES
PY1 (POSTEL) 5' CCTTCCCCACCCTCCCCACCCTCCCCA 3' 3' (AP)KY10016 2
3' CCTTCCCCACCCTCCCCACCCTCCCCA 5' (P)KY10025 3
3' NNTTNNNNANNNTNNNNANNNTNNNNA 5' (P)KY10026 4
5' CCCTCCCCACCCTCCCC 3' KY10031 5
5' CCCCACCCTCCCCACCCT 3' KY10034 6
5' iCCX.C..T 3' KY1004227
5' TTCCCCACCCTCCCCACCCTCCCCATAA 3' KY1004326
C SCRAMBLE 5'CCACCTCCCACCTCCCACCTCCCACCC 3' KY1003228
C18 5' CCCCCCCCCCCCCCCCCC 3' KY1004429
Several oligonucleotides were tested and compared, as indicated in Table II. The oligonucleotides of the present invention demonstrated an unanticipated superiority over a conventional triplex-forming oligonucleotide, specifically,
PU1 (SEQ ID NO: 30).

In contrast to the previously published results,
Postel et al., sura, C-rich oligonucleotides, and not G-rich oligonucleotides, exhibited a remarkable inhibition of thymidine uptake in HL60 cells. In the presence of lipofectin, substantial inhibition of thymidine uptake was observed at 1 pM of C-rich oligos. Moreover, the inhibitory effect was shown to be saturated at a low concentration of Crich oligos, as seen in Figure 4a. Even in the absence of lipofectin, C-rich oligos exhibited inhibition of thymidine uptake in HL60 cells at a lower concentration than the G-rich oligos. C-rich oligonucleotides also exhibited a dosedependent inhibition of thymidine uptake in K562 cells, as seen in Figure 4b.

The inhibition of cell growth by C-rich oligonucleotides was also demonstrated by cell counts. The cell counts of both HL60 and K562 are provided in Table III.

Cells were incubated with 1 ssM oligonucleotide in lipofectin as described above and counted

upon harvest. Viable cells only, as measured by Trypan blue exclusion, were counted.

Cells treated with G-rich oligonucleotides doubled their number in a 24 hour period post-treatment, while cells treated with C-rich oligonucleotides did not show any increase in the cell number after 24 hours.

TABLE III
HL60 AND K562 CELL NUMBERS AFTER TREATMENT
WITH OLIGONUCLEOTIDES AT 1 M HLgO HL0 b K562 OLIGO 10 cells/ml 10 cells/ml SEQ ID NO:
KY10013 1.63 2.02 30
KY10016 0.97 1.09 2
KY10025 0.92 1.41 3
KY10027 1.98 2.31 31
KY10032 1.18 1.40 28
No treatment 2.09 2.02 aSequences of oligonucleotides are shown in Table II.

bInitial cell number was 1x106/ml

EXAMPLE 2
Inhibition of Cell Growth in LS18O and Hut17 Cells by Oligonucleotides

A quantitative calorimetric assay for mammalian cell survival and cell proliferation was utilized for quantifying cells from the two adherent cell lines LS180 and HuH7. The assay is dependent on the reduction of tetrazolium salt MTT by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. The assay measures cell respiration and the amount of formazan produced, which is proportional to the number of living cells present in culture. In addition to unmodified phosphodiester oligonucleotides as listed in Table II, phosphorothioate oligonucleotides of the same sequence were tested. Phosphorothioate oligonucleotides exhibited much better inhibitory effect in cellular proliferation in comparison to unmodified phosphodiester.

Several C-rich phosphorothioates exhibited a drastic reduction in cell proliferation for both HuH7 and LS180 cell lines as shown in Figures 7 and 8. The "PT" following the oligonucleotide designation represents phosphorothioate. A single dose administration of C-rich oligonucleotide at 100200 nM resulted in a decrease of over 50% in cell number.

This effect was observed consistently over the five-day incubation period. In contrast, the G-rich phosphorothioate did not have an appreciable effect on cell number even at higher concentration. The C-rich phosphorothioates exhibited more drastic and prolonged inhibitory effects in cell proliferation for the period of 5 days, as compared to their phosphodiester counterparts. Controls consisting of oligonucleotide 8-mers (designated "KYlOON8-PT" in Figures 7 and 8) were synthesized using equal amounts of A, G, C, and T at each position and exhibited no effect.

As can be seen from the foregoing, the oligonucleotides of SEQ ID NOS: 2, 3, 5, 6, and 28,inhibited cell growth to various degrees in the cell lines tested and, thus, demonstrated therapeutic capability. Tests showed that the shorter length oligonucleotides such as the 18-mer of SEQ ID NO: 6 perform as well as the longer 27-mer of SEQ ID NO: 2. Although SEQ ID NO: 2 demonstrated inhibition, the oligonucleotides of the present invention preferably do not include this sequence. Notably, SEQ ID NO: 28, designated "C Scramble", shares homology with SEQ ID NO: 2, but with several deletions and mismatches, yet still inhibited cell growth.

EXAMPLE 3
Inhibition of Cell Growth Due to suppression in c-myc Expression

To determine whether inhibition of thymidine uptake in HL60 cells resulted from a decrease in c-myc transcription, the levels of c-myc and ss-actin transcripts were compared in

HL60 cells treated with G-rich and C-rich oligos. The cytoplasmic RNA was isolated, reverse transcribed, and amplified by PCR in a quantitative manner described above.

The level of the c-myc transcripts decreased in HL60 cells treated with C-rich oligos but not in HL60 cells treated with
G-rich oligos (Figure 5). In comparison, a relatively similar level of ss-actin mRNA was detected both in HL60 cells treated with C-rich or G-rich oligos. Thus, inhibition of thymidine uptake, and decrease in cell numbers, correlates with the decrease in c-myc mRNA. Neither was due to nonspecified cytoxicity.

EXAMPLE 4
Transcriptional Inhibitory Effect of Oligonucleotides
Does Not Correlate With the Binding Affinity of
Oligonucleotides to the Duplex Target.

The binding affinity of certain oligos to a duplex target was measured by polyacrylamide gel electrophoresis to determine whether transcriptional inhibition is due to the formation of an intermolecular triplex. Intramolecular triplets do not normally form under the conditions utilized for this testing. Consequently, any triplexes detected would presumably be intermolecular in nature, i.e., formed by the oligonucleotide and duplex target. Antiparallel G-rich oligos exhibited a high binding affinity, Kd = 10 nM, while
C-rich oligos did not bind to the duplex target under the experimental conditions. See Figure 6. Thus, the transcriptional inhibitory effect of C-rich oligos did not result from binding to the double-stranded region of the cmyc upstream sequence. However, C-rich oligos may be inhibiting transcription by binding to the putative singlestranded region of an intramolecular triplex in an antisense manner. In fact, the sequence of the oligonucleotide KY10025 (SEQ ID NO: 2) is entirely complementary, in anti-parallel orientation, to the G-rich loop in the putative H-DNA.

Another C-rich oligo, KY10025 (SEQ ID NO: 3), complementary to the G-rich loop in parallel orientation, exhibited a similar inhibitory effect in thymidine uptake in HL60 cells.

Thus, the C-rich oligonucleotides of the invention demonstrate the ability to decrease transcription, yet do not form a triplex with the target DNA. The foregoing results support the formation of H-DNA in vivo.

EXAMPLE 5
Erb-B2.

Erb-B2 is another example of a gene having a purinerich segment with substantial mirror symmetry. A potential site of H-DNA formation is exemplified by the mirror symmetry seen in SEQ ID NO: 8, which begins 69 nucleotides upstream of the transcription initiation site. Specifically, two substantially palindromic arms are located at nucleotides 1-9 and 17-25 of SEQ ID NO: 8. The overexpression of Erb-B2 is particularly associated with breast cancer. One example of an oligonucleotide of the invention targeted against Erb-B2 is that of SEQ ID NO: 13. This oligonucleotide and its derivatives may be used, for example, in the treatment of breast cancer, erythroleukemia, and sarcoma, and more generally, any disease involving the expression of Erb-B2.

EXAMPLE 6 RBV ppt (polypurine-rich tract)
Hepatitis B virus is an example of a human virus having a purine-rich segment with substantial mirror symmetry. The palindromic arms are seen in nucleotides 2-7 and 15-20 of SEQ ID NO: 9. Certain oligonucleotides of the invention are therefore potentially therapeutic in the treatment of diseases involving hepatitis B virus. One example of an oligonucleotide of the invention complementary to the purine-rich region with substantial two-fold symmetry is SEQ ID NO: 14. This oligonucleotide and its derivatives may be used, for example, in the treatment of hepatitis.

EXAMPLE 7
N-myc

N-myc is another example of a gene having a purinerich segment with substantial mirror symmetry. A potential site of H-DNA formation is exemplified by the mirror symmetry seen in the sequence listed in SEQ ID NO: 10, at nucleotides 5-14 and 21-30. N-myc has been implicated, for example, in neuroblastoma. One example of an oligonucleotide of the invention targeted against N-myc is that of SEQ ID NO: 15.

This oligonucleotide and its derivatives may be used, for example, in the treatment of neuroblastoma, and more generally, in the treatment of any disease involving the expression of N-myc.

EXAMPLE 8
HIV LTR
HIV is another example of a human virus having a purine-rich segment with substantial mirror symmetry. The palindromic arms of the LTR are seen in nucleotides 2-7 and 11-16 of SEQ ID NO: 11, which begins at position -293 in the
HIV genome. This region is characteristic of a nuclear factor activating T-cells (NFAT, Durand et al., Molecular
Cellular Biol., 1988, 8, 1715-1724) binding site. One example of an oligonucleotide of the invention targeted against the HIV LTR is that of SEQ ID NO: 16. This oligonucleotide and its derivatives may be used, for example, in the treatment of HIV infection.

EXAMPLE 9
Epithelial Growth Factor Receptor (EGFR)
EGFR is another example of a gene having a purine-rich region with substantial mirror symmetry. The palindromic arms are evident in nucleotides 9-17 and 25-33 of SEQ ID NO: 12, which represents an area of the gene beginning at position -363. One example of an oligonucleotide of the invention targeted against EGFR is that of SEQ ID NO: 17.

This oligonucleotide and its derivatives may be used, for example, in the treatment of lung cancer, and other cancers involving fast-growing solid tumors and, more generally, any disease involving the expression of EGFR.

EXAMPLE 10
K-ras
K-ras also exemplifies substantial mirror symmetry in an upstream transcriptionally important region that is purine-rich. The palindromic arms are visible in nucleotides 9-20 and 24-35 of SEQ ID NO: 18, which murine sequence has been published in Pestov et al., Nuc. Acids Res. 1991, 19, 6527-6532. One example of an oligonucleotide of the invention targeted against this region of K-ras is exemplified by SEQ ID NO: 19. This oligonucleotide and its derivatives may be used, for example, in the treatment of sarcoma and erythroleukemia, solid tumors, and any cancer or other disease involving the expression of K-ras.

EXAMPLE 11
IL2
IL2 has been implicated in autoimmune disorders, such as AIDS, and the upstream regulatory region of this gene likewise illustrates an area of substantial mirror symmetry in a purine-rich region. The two palindromic arms can be seen in nucleotides 4-8 and 10-14 of SEQ ID NO: 20, which represents an area of the gene beginning at position -292.

This region is characteristic of an NFAT binding site. An oligonucleotide of the invention targeted to this region is illustrated by SEQ ID NO: 21. This oligonucleotide may be used, for example, in the treatment of HIV infection other diseases involving autoimmunity and diseases requiring transplantation and, more generally, any disease involving the expression of 1L2.

EXAMPLE 12
HSV I
Herpes Simplex Virus I (HSV I) is another example of a human virus having a purine-rich segment with substantial mirror symmetry. The palindromic arms are seen in nucleotides 17-22 and 28-33 of SEQ ID NO: 22, which begins at position -60 in the HSV I genome. This

sequence represents a promoter region for the genes encoding viral polymerase and the DNA binding protein. One example of an oligonucleotide of the invention targeted against this region is that of SEQ
ID NO: 23. This oligonucleotide and its derivatives may be used, for example, in the treatment of HSV I infection.

EXAMPLE 13
HIV polypurine tract (ppt)
The polypurine-rich tract of HIV present in the 3'
Long Terminal Repeat (LTR) is also an example of a transcriptionally important region having substantial mirror symmetry. The HIV ppt is an example of a sequence in which the palindromic arms are not identical. The palindromic arms can be seen in nucleotides 11-19 and 24-32 of SEQ ID NO: 24.

One example of an oligonucleotide of the invention targeted against this region is that of SEQ ID NO: 25. This oligonucleotide may be used, for example, in the treatment of
HIV infection.

Given the present disclosure, one skilled in the art would be able to create oligonucleotides targeted against additional diseases involving genes having a region of DNA that is significantly involved in transcription, having at least about 10 nucleotides, at least about 65% of which are purines on one strand, and further having substantial mirror symmetry in at least two palindromic arms of at least about 5 nucleotides each, the arms being either contiguous or having about 1 to about 50 nucleotides between them.

One skilled in the art will recognize that application of these methods is readily generalized to any qualifying target gene and is limited only by the availability of DNA sequence data and a rudimentary understanding of the molecular genetics of the targeted gene. At present, there is a vast amount of information available that encompasses several hundred human genes and several thousand genes from other species. Such sequence information can be obtained from databases such as GenBank (IntelliGenetics, Mountain
View, CA).

SEQUENCE LISTING (1) GENERAL INFORMATION:
(i) APPLICANT: Kyonggeun Yoon and Meiqing Lu
(ii) TITLE OF INVENTION: Gene Regulation by Targeting Putative
Intramolecular Triple Helix
(iii) NUMBER OF SEQUENCES: 35
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Woodcock Washburn Kurtz
Mackiewicz & Norris
(B) STREET: One Liberty Place - 46th Floor
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19103
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: WORDPERFECT 5.1
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/008,897
(B) FILING DATE: January 25, 1993
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Doreen Yatko Trujillo

(B) REGISTRATION NUMBER: 35,719
(C) REFERENCE/DOCKET NUMBER:
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (215) 568-3100
(B) TELEFAX: (215) 568-3439 (2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
TGAGTCTCCT CCCCACCTTC CCCACCCTCC CCACCCTCCC CATAAGCGCC 50
CCTCCC 56 (2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CCTTCCCCAC CCTCCCCACC CTCCCCA 27 (2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
ACCCCTCCCA CCCCTCCCAC CCCTTCC 27 (2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1-2, 5-8, 10-12, 14-17, 19-21, 23-26
(D) OTHER INFORMATION: /label = methylcytosine
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
ANNNNTNNNA NNNNTNNNAN NNNTTNN 27 (2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
CCCTCCCCAC CCTCCCC 17 (2) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
CCCCACCCTC CCCACCCT 18 (2) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
GGTTGGGGTG GGTGGGGTGG GTGGGGT 27 (2) INFORMATION FOR SEQ ID NO: 8:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
AGGAGAAGGA GGAGGTGGAG GAGGAGGG 28 (2) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
GGGAGGAGCT GGGGGAGGAG ATTA 24 (2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
CCGCTTTCCT CTCCTTTCTC CCTCCCCCTT G 31 (2) INFORMATION FOR SEQ ID NO:
11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
AGAGAAGGTA GAAGAGGCCA AT 22 (2) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
TTCTCCTCCC TCCTCCTCGC ATTCTCCTCC TCCTCT 36 (2) INFORMATION FOR SEQ ID
NO: 13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:
TCCTCCTCCA CCTCCT 16 (2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
CTCCTCCCCC A 10 (2) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:
TTTCTCCCTC CCCCTT 16 (2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:
CATCTTCTC 9 (2) INFORMATION FOR SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:
CGCATTCTCC TCCTCC 16 (2) INFORMATION FOR SEQ ID NO: 18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:
CAGCCGCTCC CTCCCTCCCT CCTTCCCTCC CTCCCGCGCG CGCGG 45 (2)
INFORMATION FOR SEQ ID NO: 19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
CCTTCCCTCC CTCCCG 16 (2) INFORMATION FOR SEQ ID NO: 20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:
AAGAAAGGAG GAAAAACTGT TT 22 (2) INFORMATION FOR SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
TTTCCT 6 (2) INFORMATION FOR SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
TTTTTTCTCT TCCCCCCTCC CCACATTCCC CTCTTT 36 (2) INFORMATION FOR SEQ ID
NO: 23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:
ACATTCCCCT C 11 (2) INFORMATION FOR SEQ ID NO: 24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double Stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
TTTAAAAGAA AAGGGGGGAC TGGAAGGGCT AA 32 (2) INFORMATION FOR SEQ ID NO: 25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
TTAGCCCTTC CAG 13 (2) INFORMATION FOR SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:
TTCCCCACCC TCCCCACCCT CCCCATAA 28 (2) INFORMATION FOR SEQ ID NO: 27:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:
CCCCACCCT 9 (2) INFORMATION FOR SEQ ID NO: 28:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:
CCACCTCCCA CCTCCCACCT CCCACCC 27 (2) INFORMATION FOR SEQ ID NO: 29:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
CCCCCCCCCC CCCCCCCC 18 (2) INFORMATION FOR SEQ ID NO: 30:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
GGTTGGGGTG GGTGGGGTGG GTGGGGT 27 (2) INFORMATION FOR SEQ ID NO: 31:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
GGTGGAGGGT GGAGGGTGGA GGGTGGG 27 (2) INFORMATION FOR SEQ ID NO: 32:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:
AGCAGCGACT CTGAGGAGGA ACAAGAA 27 (2) INFORMATION FOR SEQ ID NO: 33:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:
AGCAGGATAG TCCTTCCGAG TGGA 24 (2) INFORMATION FOR SEQ ID NO: 34:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:
GCATGGAGTC CTGTGGCATC CACG 24 (2) INFORMATION FOR SEQ ID NO: 35:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single Stranded
(D) TOPOLOGY: Linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:
CTAGAAGCAT TTGCGGTGGA CGAT 24